Full Length Research Paper

Gene expression and antioxidant enzymatic activity in passion fruit exposed to aluminum

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The objective of this study was to investigate changes in enzymatic activity and gene expression related to antioxidant defense in two passion fruit cultivars (FB100 and FB200) exposed to aluminum. The specific activity of superoxide dismutase (SOD), catalase and ascorbate peroxidase were analyzed, in addition to their relative expression pattern. The characterization of gene expression was achieved by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) technique followed by densitometric analysis. Plants were irrigated every two days with a nutrient solution: pH 6; pH 4; 0.2 and 2.0 mM Al (pH 4). The leaves were collected at the times 0 h, 2 days, 5 days and 10 days. The analysis of the potential oxidative stress induced by aluminum demonstrated increased expression of catalase and superoxide dismutase gene, and only on the cultivar FB100 this increase was accompanied by an increase of SOD enzymatic activity, which indicates its higher efficiency on the removal of reactive oxygen species (ROS) compared to cultivar FB200.

Key words: oxidative stress, *Passiflora edulis*, aluminum, semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), differential expression.

INTRODUCTION

Aluminum (Al) present in soil solution is one of the main agents responsible for reduced productivity in plants (Panda and Matsumoto, 2007). Recent studies have shown that this effect is explained by the inhibition of root growth as well as the reduction of water absorption. Also, how morphological, biochemical and physiological changes occur in different plants was investigated (Sevik and Cetin, 2015; Kravkaz-Kuscu et al., 2018). Meanwhile, Al is the most abundant metal in the earth's crust, and at micromolar concentrations, it causes morphological, biochemical and physiological changes in plants. Toxicity caused by aluminum is exacerbated for plants grown in acidic soils, especially at pH below 5. In acidic solutions, Al is present as Al^{3+}, characteristically toxic to plants (Giannakoula et al., 2008). Over 70% of the world's soil is contaminated with acid, alkali and heavy metals. However, acid soil is the factor that most limits the production of staple foods in the world. Approximately

50% of arable land is negatively impacted by Al toxicity due to acid soil (Panda et al., 2009). The Al toxicity has been further aggravated by the use of fertilizers and acid rains (Singh et al., 2017).

Aluminum induces the formation of reactive oxygen species (ROS). Evidence that supports this hypothesis is guided on correlations established between exposure to Al and increased ROS, higher activity of some enzymes and/or concentration of metabolites (Darkó et al., 2004). ROS accumulation causes oxidative damage and alters cell functions. Plants have developed defense systems against ROS, including the synthesis of enzymes such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (Ahmad et al., 2010). The increased activity of antioxidant enzymes is also reported as being a mechanism for tolerance to Al in some varieties of corn and wheat (Boscolo et al., 2003; Ma et al., 2007). Changes in the activity of antioxidant enzymes are an important metabolic process that can influence on tolerance to aluminum by higher plants (Ezaki et al., 2013). Such changes depend on the plant species, the intensity and duration of stress.

Studies indicate increased gene expression encoding antioxidant defense system proteins upon exposure to Al. In *Arabidopsis thaliana* it was observed that aluminum induces gene expression related to oxidative stress, which constitutes an important component of plant responses to toxic levels of aluminum (Corrales et al., 2008) by causing the activation of antioxidant enzymes. The semi-quantitative PCR technique can be used as a tool to study gene expression. This technique is based on comparing the levels of amplification in PCR's with different numbers of cycles, becoming a rapid and reliable way of quantifying specific mRNA present in small quantity in the sample (Rey et al., 2000). The implications of Al toxicity for oxidative stress have been widely investigated in roots of different plant species (Boscolo et al., 2003). However, little information is available about the damage induced by Al in foliar tissues. In addition, few studies have linked the pattern of gene expression and antioxidant enzyme activity related to tolerance to aluminum. The objective of the study was to investigate possible changes in enzyme activity and gene expression, in transcriptional and translational level, respectively, on passion fruit leaves exposed to Al.

MATERIALS AND METHODS

Plant material and experimental design

The experiment was conducted in a greenhouse located in the Goiabeiras campus at Federal University of Espírito Santo/UFES (20°18'52" S and 40°19'06" W). In the experiment were used seeds of *Passiflora edulis* SIMS *cv.* FB 100 ("Maguary") and FB200 ("Yellow Master"). Plants were watered every two days with 200 ml of a modified nutrient solution (Hoagland and Arnon, 1950) half strength, at 28°C (± 2) and natural photoperiod. Stress induction began 45 days after transplanting and lasted 10 days. The treatments were: control (pH 6); pH (pH 4); 0.2 mM Al^{3+} (pH 4) and

2 mM of Al^{3+} (pH 4). Al was added as $AlCl_3$. The leaves were collected at the times 0 h, 2, 5 and 10 days, after starting treatment with Al, instantly frozen in liquid nitrogen and stored at -80°C.

Enzymatic activity

0.2 g of tissue leaf with 2% (w/v) polyvinylpolypyrrolidone (PVPP) were macerated. Then, was added 1500 µl of extraction solution containing: 750 µl of 100 mM potassium phosphate buffer (pH 6.8), 15 µl of 10 mM EDTA, 150 µl of 100 mM ascorbic acid and 585 µl of ultrapure water. Cell residue was removed by centrifugation for 15 min at 13,000 g at 4°C. This extract was used in the analysis of enzymatic activity and quantification of total soluble protein. For determining total soluble protein contents, the method proposed by Bradford (1976) was used. The crude catalase activity was calculated based on the molar extinction coefficient of H_2O_2 (0.036 µmol cm^{-1}) (Anderson et al., 1995). To determine the crude ascorbate peroxidase activity, the molar extinction coefficient of ascorbate of 0.0028 $µmol^{-1}$ cm was used (Nakano and Asada, 1981). The crude superoxide dismutase activity an enzyme reaction medium was determined according to Beauchamp and Fridovich (1971). The specific activity of CAT, APX and SOD was determined by dividing the crude enzymatic activity by the content of total soluble proteins. The analyses were performed with 5 replicates.

RNA extraction

RNA extraction of collected leaves was performed at time zero, 5 and 10 days. Approximately 100 mg of powdered plant tissue with liquid nitrogen was stored at -80°C. A repetition scheme was organized in 3 pools, each containing 4 different samples. For total RNA extraction, TRIzol Reagent (Invitrogen®) was used, according to manufacturer's recommendations. The resulting RNA was quantified in a NanoDrop 3300 Termo Scientific spectrophotometer. RNA samples were treated with DNAse I AmpGrade (Invitrogen®).

Semi-quantitative RT-PCR

Extracted RNA was used for RT-PCR (Reverse Transcription Polymerase Chain Reaction) having as control the constitutive 18S ribosomal gene. The first complementary DNA strand (cDNA) was synthesized with the kit GoScript™ (Promega®), according to the manufacturer's recommendations. The cDNA samples were quantified using NanoDrop 3300 (Termo Scientific). For the initial amplification of DNA from cDNA fragments obtained, primers were used for each gene that would be analyzed. The primers used for gene expression of *P. edulis* were as follows: 18s 5'-TGACGGAAGAATTAGGGTTCG-3'   5'-GACTTGCCCTCCAATGGATC-3' (Azevedo et al., 2003), Cat 5′-CTGCTGGAAACTATCCTGAGTG-3′   5′-ATTGACCTCTTCATCCCTGTG-3′ (Balestrasse et al., 2008) and Sod   5′-CTACGTCGCCAACTACAACAAG-3'   5′-GTAGTACGCATGCTCCCAGAC-3' (Baek and Skinner, 2005).

From the cDNA fragments obtained, specific primers were designed for each gene using Primer 3 and Gene Runner software. Primers designed: Cat F 5′-GTCAACCCGCAAACCACAA-3′ R 5′-ACACCCATAGGCACCGTCT-3′ Tm 54°C and Sod F 5′-TCCAACCCGATAACTGTG -3' R 5′-TCTGGTGACGAAGGGAGC -3' Tm 53°C. Each of the primers was subjected to reaction with 32, 25, 20, and 15 cycles of denaturation, annealing and extension. The amplified products were subjected to 2% agarose gel run, stained with GelRedTM (BIOTIUM™), visualized by UV transilluminator and DNA bands analyzed using ImageJ software. The DNA fragments were sequenced to confirm their identity, by alignment with sequences deposited in GeneBank (NCBI) using the

BLAST program (Basic Local Alignment Search Tool - NCBI). Sequencing was performed using automated DNA sequencer Applied Biosystems model 310.

Statistical analysis

The results related to parametric data were submitted to analysis of variance (ANOVA) and the means were compared by Tukey test at 5% probability.

RESULTS AND DISCUSSION

Aluminum at toxic levels can induce damage caused by oxidative stress, as a result of increased production and accumulation of ROS (Pereira et al., 2010). They control vital processes in plant organisms, however as toxic molecules can cause injuries on the cells, such as the oxidation of lipid membranes, proteins and nucleic acids, as well as interference in physiological processes such as photosynthesis and respiration (Scandalios, 2005). Among the enzymes of the antioxidant system, catalase convert H_2O_2 into molecular oxygen and water, but have low affinity to the substrate. The catalase enzyme did not have its activity changed by the treatments (Figure 1A and B). Boscolo et al. (2003) also reported that there were no changes in CAT activity in corn exposed to aluminum toxicity. This same CAT response was observed in *Oryza sativa* L. under light stress (Aumonde et al., 2013).

The radical considered more reactive to catalase is O_2^-, which can easily move across membranes, thus representing the main inhibitor of this enzyme (Scandalios, 2005). SOD catalyzes the degradation reaction from O_2^- to H_2O_2, in contrast, high levels of this product inhibit its catalytic action. There was an increase in SOD activity in low aluminum concentration only in the cv. FB100, and when the plants were exposed to 2 mM Al there was a return of SOD activity to basal levels equating to control (Figure 1E and F). Pereira et al. (2010) observed increase in SOD activity in *Cucumis sativus* at low aluminum concentration. However, at high concentrations (2 mM), these authors observed decrease in SOD activity, a result similar to that found in this study. The decrease in SOD activity in *P. edulis* may indicate excess peroxide in the leaf tissue, since there was no increase in CAT and APX activity. Peroxide is able to inactivate enzymes by oxidation of the thiol groups. In addition, peroxide is a very stable ROS and its increased synthesis has been shown as a response to various types of stresses (Apel and Hirt, 2004).

APX, as well as CAT, also convert peroxide into O_2 and water, but do so using ascorbate as an electron donor, representing an alternative way of destruction of H_2O_2 (Pereira et al., 2011). In this study, both cultivars maintained APX activity similar to control (Figure 1C and D). González-Santana et al. (2012) also observed that there was no change in APX activity in leaves and roots of *Conostegia xalapensis* exposed to aluminum. Similarly, Panda et al. (2010) showed that APX activity showed no difference in *Pisum sativum* root exposed to aluminum.

Studies show that Al can induce gene expression against its toxic effect; these genes are mostly responsible for the production of enzymes related to antioxidant defense system of plants (Corrales et al., 2008). The DNA sequence obtained from cDNA amplification with primer 18S showed similarity of 99% to 18S ribosomal RNA sequences for 48 species of plants. The cDNA sequence obtained from the primer *Cat* (329pb) showed a high percentage of homology with genes from another species: 80% with *Cat1* gene and *Cat3* of *Brassica juncea*. The cDNA sequence obtained from the specific *Sod* primer (172pb) had homology with the *MnSOD* gene of the species: 88% with *Eichhornia crassipes* and 85% with *Prunus persica*. This homology comparison proves that the primers designed for this study were actually *Cat* and *Sod* gene primers, confirming that the quantified material corresponds to the sequences of the enzymes investigated.

The CAT enzyme is directly involved in controlling damage caused by H_2O_2. The study of gene expression verified that PCR of 25 cycles, the level of transcription of *Cat* cv. FB100 and cv. FB200 was found to be elevated with 2 mM Al, at 5 and 10 days (Figure 2). On the PCR of 15 cycles, *Cat* amplification was reduced, preventing the densitometric analysis. *Cat* transcription levels indicate that the FB100 cultivar is more efficient in the removal of ROS, since at the higher concentration of Al there was increase in production of *Cat* transcripts, at 5 days. In contrast, in the FB200 cultivar that increase in *Cat* transcripts occurred after 10 days, which demonstrates that the cultivar FB100 presented a faster EROS removal response. Induction of gene transcription after exposure to stress factors has also been reported by Balestrasse et al. (2008) where elevated levels of *Cat* transcripts occurred in soybean nodules under cadmium stress. For *Ulva fasciata*, Sung et al. (2009) reported an increase in the amplification of *Cat* genes after imposed to salt stress.

The results obtained with relative expression of *Sod* cv. FB100, with 25 cycles, show that amplification was greater in the plants treated with aluminum (Al 0.2 mM and 2 mM) in the 5-day samples, as for 10-day samples only the treatment with 2 mM Al exhibited higher levels of transcripts. For cv. FB200, the level of expression was increased in the treated plants with lower aluminum concentration (0.2 mM) only at 10 days. On the PCRs of 20 and 15 cycles, densitometric analysis was not possible by reduced amplification (Figure 3). *Sod* gene expression was increased by treatment with aluminum using concentration of 2 mM Al at 5 and 10 days in the cv.FB100, and this differential expression was accompanied by an increase in the enzymatic activity of SOD in the same cultivar. Panda et al. (2010) reported an
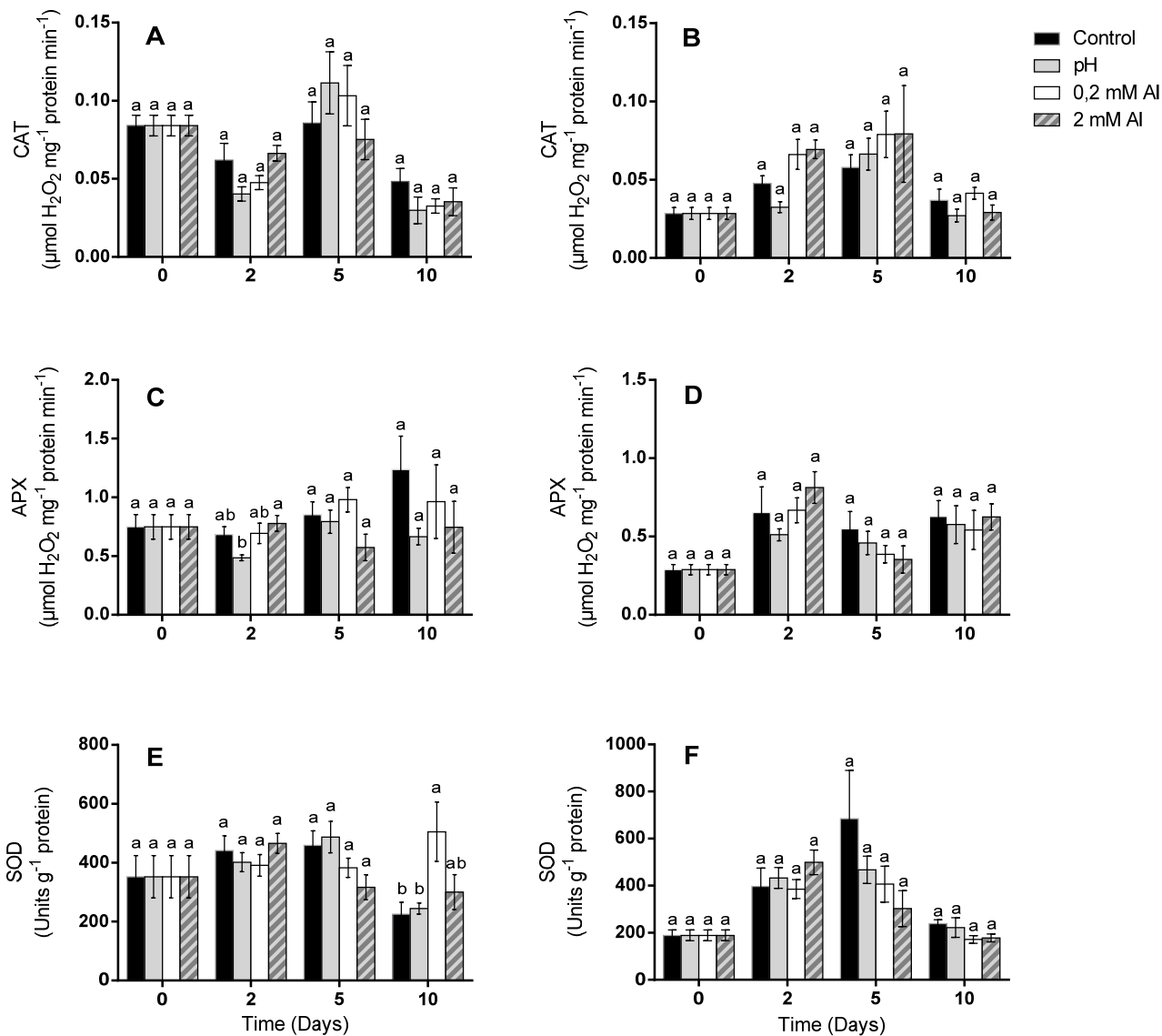
Figure 1. CAT enzymatic activity cv FB100(A) and cv FB200 (B); APX cv FB100(C) and cv FB200 (D); SOD cv FB100(E) and cv FB200 (F) in *P. edulis* leaves. The results represent the mean ± SE. Different lowercase letters indicate statistical significance by Tukey test (p <0.05).

increase in expression level of different types of *Sod* in *Pisum sativum*. Bhoomika et al. (2013) identified that both *FeSod* and *MnSod* participate in stress tolerance by aluminum toxicity in rice cultivar tolerant to this type of stress. *Sod* are the first line of defense against ROS. As the O_2^- radical is produced in any intracellular location where there is transport of electrons, the activation of O_2 can occur in different cellular compartments. Tolerant cultivars tend to increase their antioxidant enzyme activity as well as the induction of genes that encode such enzymes (Panda et al., 2010; Pereira et al., 2010). Thus, elevated levels of *Sod* and *Cat* transcripts accompanied by the increase in the enzymatic activity of SOD, only on cv. FB100 may indicate that it presents more efficient
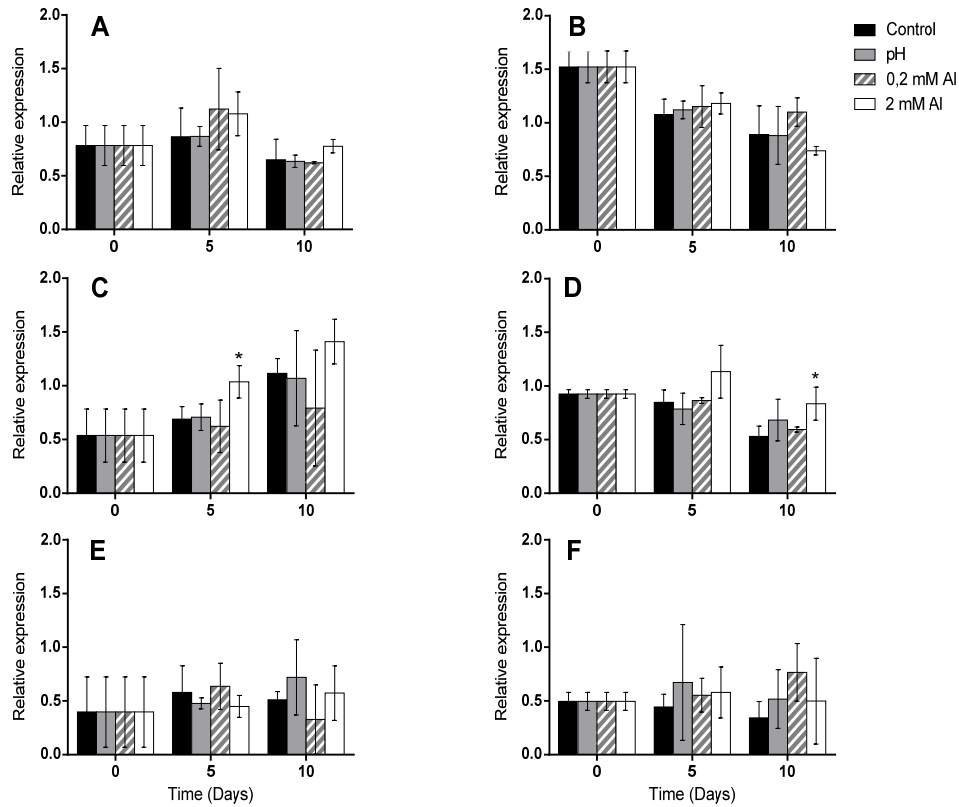
antioxidant defense system, which gives it greater tolerance to aluminum stress when compared to cv. FB200.
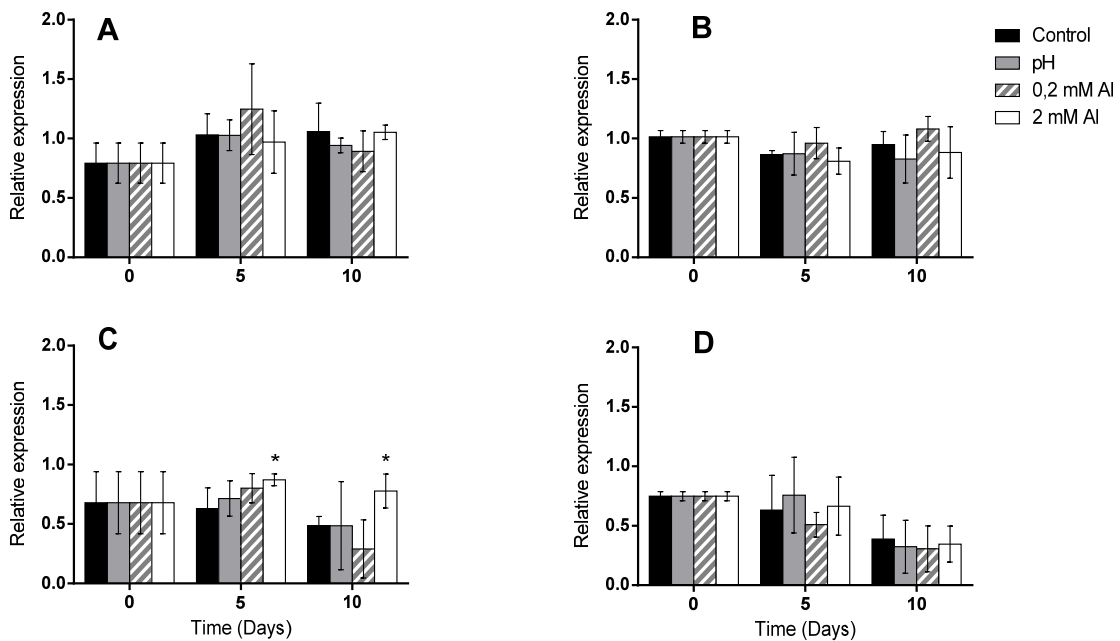
CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Figure 2. *Cat* relative expression of *P.edulis* PCR with 32 cycles cv FB100 (A) and cv FB200 (B); 25 cycles cv FB100 (C) and cv FB200 (D) and 20 cycles cv FB100 (E) and cv FB200 (F). The results represent the mean ± SD. Asterisk means the value is significantly different from the control.

Figure 3. *Sod* relative expression of *P.edulis* PCR: 32 cycles cv FB100 (A) and cv FB200 (B); 25 cycles cv FB100 (C) and cv FB200 (D). The results represent the mean ± SD. Asterisk means the value is significantly different from the control.

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